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Regulation of phosphate transport in proximal tubules

J. Biber · N. Hernando · I. Forster · H. Murer

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Abstract Homeostasis of inorganic phosphate (P_i) is primarily an affair of the kidneys. Reabsorption of the bulk of filtered P_i occurs along the renal proximal tubule and is initiated by apically localized Na^+-dependent P_i cotransporters. Tubular P_i reabsorption and therefore renal excretion of P_i is controlled by a number of hormones, including phosphatonins, and metabolic factors. In most cases, regulation of P_i reabsorption is achieved by changing the apical abundance of Na^+/Pi cotransporters. The regulatory mechanisms involve various signaling pathways and a number of proteins that interact with Na^+/P_i cotransporters.

Keywords Sodium-dependent cotransport · Phosphate homeostasis · Regulation · Phosphate excretion

Introduction

If one considers the diversity of biological functions of inorganic phosphate (P_i,) it should come as no surprise that an organism requires mechanisms to maintain the extracellular P_i reasonably constant. Deviations of the extracellular concentration of P_i will impair a variety of physiological processes such as bone metabolism, cellular energetics, protein synthesis, and signaling cascades. In humans, both hypo- and hyperphosphatemia have severe clinical consequences that underscore the need for stable and well-managed P_i homeostasis [51, 62].

In mammals, including humans, plasma concentration of P_i is determined by the intestinal intake of P_i, the excretion of P_i via the feces, the release of P_i from bone and soft tissue, and the renal excretion of P_i. It is the latter mechanism that is of greatest importance for whole body homeostasis of P_i and therefore needs to be tightly controlled. In quantitative terms, human kidneys filter approximately 200 mmol (or ~20 g) of P_i per day. In the steady state, approximately 15% or 30 mmol (~3 g) P_i of the filtered load is excreted by the kidneys. Under physiological equilibrium conditions, the total daily renal and fecal P_i excretion is approximately equal to the amount of intestinally absorbed P_i for a normal diet. However, renal excretion of P_i does not always operate at a constant rate, but it is permanently adjusted according to the daily fluctuations of numerous hormones, including the recently described phosphatonins, and metabolic factors [11, 108, 12] (Table 1). This implies that the transporters involved in reabsorbing P_i in the proximal tubule are subject to regulatory pathways that (in most cases) affect the abundance of apical Na^+/P_i cotransporters [84, 108]. Moreover, growth and age as factors affecting renal P_i handling must also be considered as potential regulators of P_i transporters [110].

This review focuses on those Na^+/P_i cotransporters localized in the apical membrane of proximal tubules that are involved in the renal P_i reabsorption and which respond to P_i homeostatic regulatory factors. In this context, we will concentrate on the mechanisms that regulate P_i excretion via modification of apical expression of Na^+/P_i cotransporters under both normal physiological and pathophysiological conditions. As most of the data comes from studies on rodents, one should exercise caution when extrapolating

J. Biber · N. Hernando · I. Forster · H. Murer
Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

J. Biber (✉)
Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland
e-mail: JuergBiber@access.uzh.ch

Table 1 Factors affecting proximal tubular phosphate reabsorption

Reabsorption decreased by	Reabsorption increased by
Parathyroid hormone	Growth hormone
Dopamine	Insulin-like growth factor
Phosphatonins (FGF-23; sFRP-4; MEPE)	$1,25(OH)_2D3$
Glucocorticoids	Phosphate depletion
Atrial Natriuretic Peptide	
Phosphate loading	
Metabolic acidosis	
Carbonic anhydrase inhibitors	
Estrogen	

the conclusions to higher mammals, including humans. For more integrated views of whole body P_i homeostasis and specific details of genetic and acquired diseases that impair renal P_i handling, the reader should refer to the following articles [14, 17, 55, 70, 91, 108, 113, 117, 126].

Localization and composition of the renal machinery for P_i reabsorption

The P_i concentration in the glomerular ultrafiltrate is approximately equal to that in the plasma. Under normal physiological conditions, approximately 80% of P_i contained in the primary urine is reabsorbed unidirectionally along the proximal tubules. The amount of P_i reabsorbed in proximal convoluted tubules is up to threefold higher than that in proximal straight tubules. There is no evidence for reabsorption of P_i along the loop of Henle, but some P_i may be absorbed along distal tubular segments; however, the identity of the distal tubular transporters is unknown. Moreover, it is unclear if P_i handling in the distal nephron is relevant for the systemic control of P_i [for review see, 11, 108].

Uptake of P_i at the brush border membrane of proximal tubular cells is strictly dependent on the presence of Na^+ ions [121], which indicates that there is no paracellular transport of P_i via cell–cell contact sites. Indeed, all P_i transporter proteins identified so far and that are localized at the brush border membrane of proximal tubule cells are Na^+-dependent. They mediate secondary-active transport by coupling to the free energy of the transmembrane Na^+ gradient that is maintained by the Na^+/K^+-ATPase localized at the basolateral membrane (Fig. 1). In theory, this would allow sufficient accumulation of P_i within the cell to provide the driving force for the exit step(s) of P_i at the basolateral side. No detailed information is currently available about the intracellular concentration of free P_i or the molecular identity of P_i transporters localized at the basolateral membrane.

It has been established that Na^+/P_i cotransport through the brush border membrane is mediated chiefly by gene products of the solute carrier family SLC34 [83]; designated SLC34A1 (NaPi-lla) and SLC34A3 (NaPi-llc). Both SLC34A1 and SLC34A3 are localized at the brush border membrane of proximal tubule cells and have not been detected in other segments of the nephron [32, 99]. Under normal dietary conditions, these proteins are most abundant in S1 segments of both cortical and juxtamedually nephrons, with the highest abundance in the latter. In contrast to NaPi-lla, which gradually decreases along the entire length of the proximal tubule, NaPi-llc has not been detected in the S3 segments of rat and mouse kidneys (Fig. 1) [32, 90].

A gene product of the solute carrier family 17 (SLC17A1 or NaPi-1) was identified based on Na^+/P_i cotransport in an expression cloning study using *Xenopus laevis* oocytes and was also localized to the brush border membranes of proximal tubules [33]. However, follow-up studies indicated that this cotransporter is most likely not involved in proximal tubular reabsorption of P_i, but rather in the transport of organic ions [21].

Finally, recent findings indicate that a gene product of the solute carrier family 20 (SLC20A2 or PiT-2) [29]) should also be considered as a potential candidate for proximal tubular P_i reabsorption, as this protein was localized exclusively at the brush border membrane of

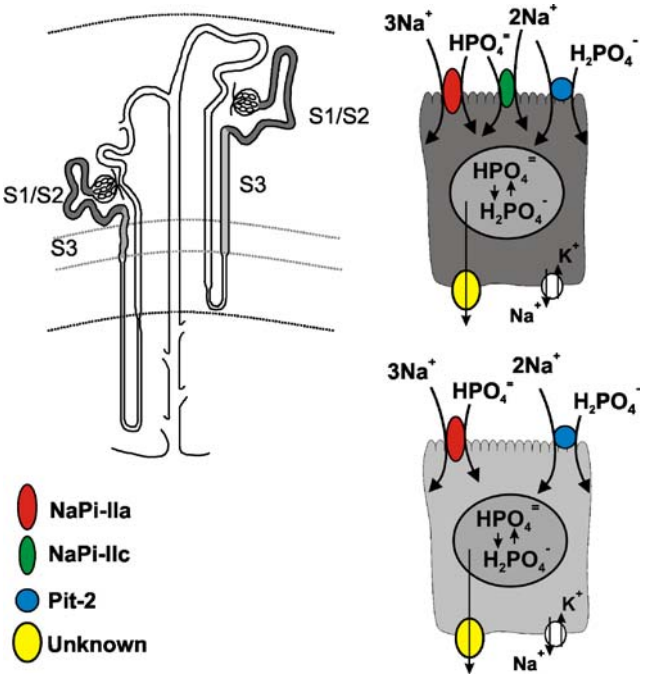

Fig. 1 The renal P_i-reabsorption machinery in rat and mouse proximal tubules. Na^+/P_i-cotransporters NaPi-lla, NaPi-llc and Pit-2 are localized at the brush borders of proximal tubules. NaPi-lla and Pit-2 proteins are detected in S1, S2 and S3 segments, whereas NaPi-llc cotransporter is absent in S3 segments

proximal tubules (Villa-Bellosta R, Ravera S, et al., unpublished observations). Its contribution to proximal tubule P_i reabsorption and significance for whole body P_i homeostasis remain to be determined.

How do different Na^+/P_i cotransporters contribute to renal P_i reabsorption?

Recent studies indicate that to answer this question, we must take account of the species and developmental stage of the animals under investigation. Furthermore, the finding that NaPi-lla, NaPi-llc, and Pit-2 show different time courses of dietary or hormonal regulation suggests that the underlying signaling pathways differ.

Knockout of the gene encoding NaPi-lla (Npt2) established that in adult mice, P_i reabsorption is mediated largely by NaPi-lla [7]. In brush border membrane vesicles (BBMV's) isolated from $Npt2^{-/-}$ mice, Na^+-dependent P_i uptake was reduced by 70%, which could account for the observed hypophosphatemic state of these animals. The remaining 30% of Na^+/P_i cotransport in BBMV's has been attributed to the activity of NaPi-llc as its abundance increased in $Npt2^{-/-}$ mice [115]. Taken together, these observations suggested that in adult wild-type mice, Na^+/P_i cotransport via NaPi-llc plays only a marginal role. In agreement, NaPi-llc abundance was found to be highest in weaning mice and decreased with age [99]. This conclusion is further supported by the lack of a phenotype in NaPi-$llc^{-/-}$ mice [79].

In humans, particularly adults, the contribution of the NaPi-IIc to renal reabsorption of P_i and to whole body P_i homeostasis appears to be more important than in mice. Mutations in the SLC34A3 gene have been described in patients with hereditary hypophosphatemic rickets with hypercalcuria (HHRH) by two independent studies [9, 75]. The renal wasting of P_i associated with this disease can therefore be correlated with an impaired function of NaPi-llc. This suggests that in adult humans, NaPi-llc contributes significantly to renal P_i reabsorption under normal steady-state conditions.

On the basis of in situ hybridization, expression of both members of the SLC20 family [29], Pit-1 and Pit-2, was detected along the entire nephron [116]. As noted above, the Pit-2 protein was recently localized in the brush border membranes of proximal tubules (Villa-Bellosta R, Ravera S et al., unpublished observations), but its contribution to renal P_i reabsorption remains unknown.

Na^+/P_i cotransporters at work

The transport kinetics of NaPi-lla, NaPi-llc, and Pit-1, -2 have been characterized in detail using the *Xenopus laevis* oocyte expression system [for review see 38, 124]. All four proteins prefer Na^+ ions as the driving substrate, and P_i as the driven substrate, yet there are important differences that could influence their respective physiological functions.

First, NaPi-IIa is electrogenic, whereas NaPi-IIc is electroneutral. This finding is consistent with their different transport stoichiometries. Both prefer divalent P_i ($HPO_4^{=}$); NaPi-lla displays a Na^+:P_i stoichiometry of 3:1, whereas for NaPi-llc, the stoichiometry is 2:1. Thus, the electrogenicity of NaPi-lla arises from the translocation of an additional Na^+ ion, at greater energetic cost to the cell. Despite this fundamental difference, at neutral pH, both NaPi-IIa and NaPi-IIc show similar apparent affinities for Na^+(~50 mM) and P_i (<0.1 mM) as well as a qualitatively similar decreased transport activity with external acidification [38, 124, 125].

Second, in contrast to NaPi-IIa/c, PiT-1/2 prefer monovalent P_i ($H_2PO_4^-$). Like NaPi-lla, both Pit-1 and Pit-2 are electrogenic, yet Pit-1 has been shown experimentally to display a 2:1 Na^+:Pi stoichiometry. Apparent affinities for Na^+ are in the range of 50 to 70 mM and for P_i~0.1 mM [18, 93, 124]. Unlike NaPi-IIa/c, Li^+ can also support P_i translocation by PiT-1, but at a significantly reduced rate and there is a weak affinity for arsenate [93].

Third, the pH dependence of NaP-lla and NaPi-llc is similar; transport rates approximately double between pH 6.5 and 8 [124]. Such a pH-dependence may have to be considered when assessing renal excretion of P_i under conditions of an altered acid base status of the body [46, 87]. In contrast, the predicted maximum transport velocity for Pit-1/2-mediated Na^+/P_i cotransport is quite constant within this pH range [93].

Finally, until recently, phosphonoformic acid (PFA) was considered a general competitive inhibitor of Na^+/P_i cotransport [112]. Current data indicate that it inhibits only Na^+/P_i cotransport mediated by SLC34 family gene products. The K_i for PFA is approximately tenfold higher for PiT-1, -2 compared to that reported for NaPi-IIa. This may be a direct consequence of the different preference for P_i of the two families as PFA is expected to be divalent under normal physiological conditions [93].

Currently, no information about the three-dimensional structure of Na^+/P_i cotransporters is available. Based on extensive studies using cysteine scanning combined with functional studies and in vitro transcription/translation approaches, SLC34 proteins are predicted to comprise eight transmembrane domains with intracellular N- and C-termini. A large extracellular loop region links two halves of the protein that contain conserved repeated sequences also found in bacterial homologs. The repeats are proposed to form opposed reentrant loops that most likely constitute the transport pathway [38, 92, 124]. The secondary structure of SLC20 proteins is less well defined: they are

predicted to comprise ten transmembrane domains with extracellular N- and C-termini, however evidence for an obvious repeat region is so far lacking, and there is no homology with the SLC34 gene products in the proposed substrate translocation region [18, 98].

The functional unit of NaPi-IIa is a monomer [63]. Interestingly, a split-ubiquitin yeast two-hybrid assay, and subsequent studies provided evidence that NaPi-IIa may homodimerize [42]. Although apparently not relevant for transport function, one can speculate that homotypic interactions of NaPi-IIa proteins may be of relevance for the regulation of this cotransporter.

Apical positioning of Na^+/P_i cotransporters

After correct targeting and insertion into the plasma membrane of epithelial cells, specific protein–protein interactions may be required to stabilize the final localization of membrane proteins [130]. Classical yeast two-hybrid screens performed against the C-terminus of NaPi-IIa revealed interactions of this cotransporter with several PDZ domain (*P*SD-95, *d*iscs-large, *Z*O-1) containing proteins that may contribute to the stabilization of NaPi-IIa at the apical membrane (Fig. 2b) [15, 42–44]. Robust interactions of NaPi-IIa with single PDZ domains of the sodium–hydrogen exchanger regulatory factor (NHERF), protein family [104], NHERF1 and 2, NHERF3 (PDZK1) and NHERF4 (PDZK2) have been demonstrated in in vitro studies. Members of the NHERF family were proposed to scaffold several other membrane proteins, including transporters and receptors as well [43, 44]. Furthermore, NHERF1/2 bind to members of the merlin–ezrin–radixin–moesin (MERM) protein family, thereby providing a link to the cytoskeleton [104].

The interactions of NaPi-IIa with PDZ domains of the NHERF family were shown to be primarily via a class I PDZ binding motif (TRL) located at the C-terminus [43, 44]. In opossum kidney (OK) cells, the TRL-motif was shown to be required for apical expression [49]. The roles of some of these interactions of NaPi-IIa with PDZ proteins have been elucidated using several mouse knockout

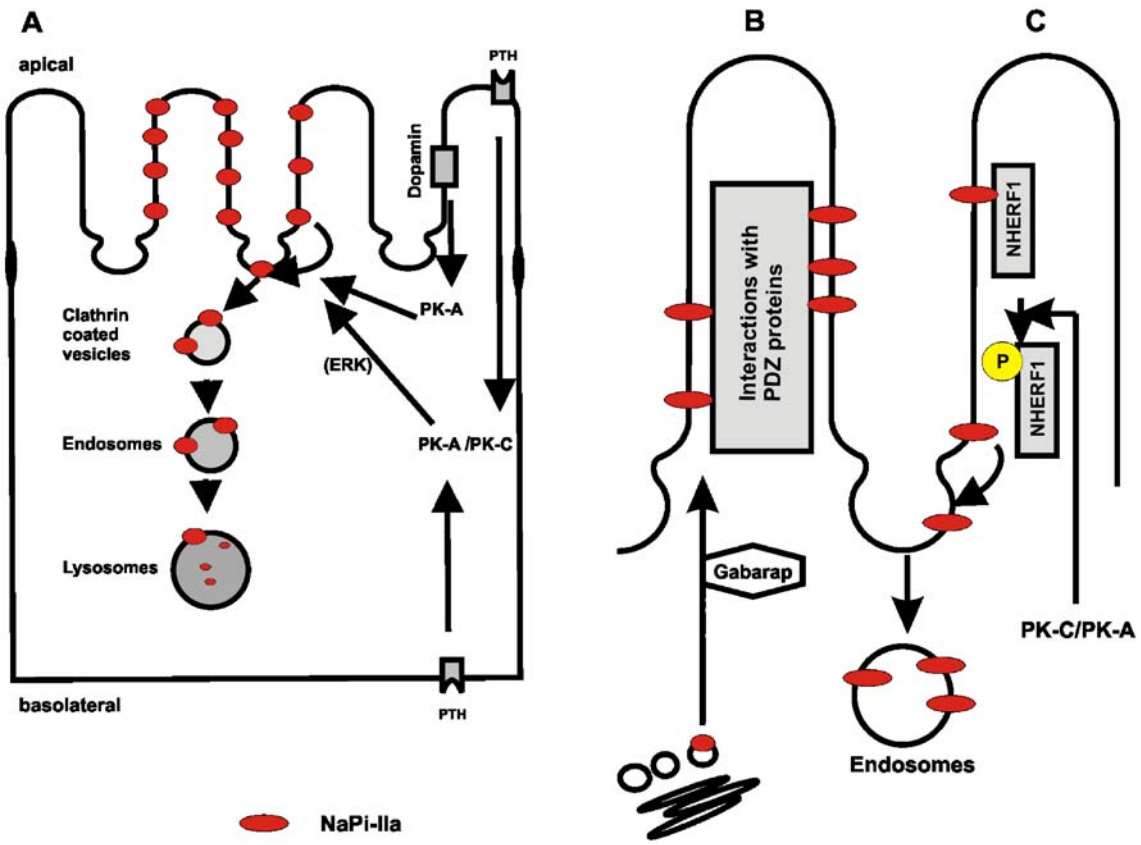

Fig. 2 Regulation of the NaPi-IIa Na^+/P_i-cotransporter. **a** Activation of protein kinase dependent signaling pathways, e.g, by PTH or dopamine, provoke internalization of NaPi-IIa cotransporters at the intermicrovillar clefts. Internalized NaPi-IIa proteins are degraded in the lysosomes. **b** Schematic illustration of the apical sorting and positioning of NaPi-IIa by PDZ proteins [15, 43, 44, 104]. The recently suggested role of Gabarap in determining apical abundance of NaPi-IIa [94] is also indicated. **c** Modulation of the interaction of NaPi-IIa with NHERF1 by protein kinases activated by PTH. Phosphorylation of NHERF1 [128] weakens the apparent affinity for NaPi-IIa, which is subsequently delivered to the endocytic machinery

models. NHERF1$^{-/-}$ mice exhibited phosphaturia that could be explained by a decrease of the apical abundance of NaPi-lla [105]. These findings indicate a stabilization effect of NHERF1 on the membrane expression of NaPi-lla. In contrast, in NHERF2$^{-/-}$ mice, urinary excretion of P_i and apical expression of NaPi-lla were unaffected under normal physiological conditions [31]. Despite the robust in vitro interactions of NaPi-lla with NHERF3, neither urinary excretion of P_i nor the abundance of NaPi-lla was impaired in NHERF3$^{-/-}$mice [23].

In addition to the NHERF proteins, NaPi-lla also interacts with the PDZ domain of Shank2E, a splice form of Shank2 [78]. Shank2E is localized at the brush border membrane of proximal tubule cells, and there is evidence that it may be involved in the endocytic machinery that determines the surface abundance of NaPi-lla.

A recent study revealed a role for the $GABA_A$-receptor associated protein (Gabarap) in determining the apical amount of NaPi-lla [94]. In BBMV isolated from Gabarap$^{-/-}$ mice, the amount of NaPi-lla was increased, whereas NaPi-llc was unaffected. In agreement with these findings, Na^+/P_i cotransport was higher in BBMV's from Gabarap$^{-/-}$ animals, which was reflected in a reduced urinary P_i excretion. The molecular mechanism underlying the action of Gabarap is unknown. Gabarap may exert its effect on he apical sorting as it interacts with the *N*-ethylmaleimide-sensitive factor NSF [61]. Alternatively, NHERF1 which was also up-regulated in Gabarap$^{-/-}$ mice [94], may increase the stabilization of NaPi-lla in the brush border membrane of these animals.

Currently, there is little information about proteins interacting with NaPi-llc or Pit-2. NaPi-IIc was reported to interact with NHERF1 and NHERF3 [123], the consequences of these interactions for apical positioning and regulation of NaPi-llc remain to be clarified.

Regulation of proximal tubular Na^+/Pi cotransport

General considerations

Theoretical considerations predict that at equilibrium, secondary active Na^+/P_i cotransport across the brush border membrane of proximal tubules would give rise to a concentrating capacity of 100:1 for NaPi-llc and 10,000:1 for NaPi-lla [45, 124]. Although precise values for the intracellular concentration of P_i are unavailable, it is estimated to be in the lower millimolar range [14]. Thus, for a given membrane protein density, the capacity of proximal epithelia to reabsorb P_i is not determined by the driving force but by the turnover rate of the transport cycle. Alteration of P_i reabsorption therefore could be achieved either by changes in the abundance of Na^+/P_i cotransporters, the apparent affinities for the substrates or the lipid composition, which could influence the preferred conformational state of the protein and modulate partial reactions that constitute the transport cycle.

Most of the known effects of hormones and metabolic factors (Table 1) on renal P_i absorption take place via alterations of the abundance of Na^+/P_i cotransporters within the proximal brush border membrane. The membrane abundance of a Na^+/P_i cotransporter depends on the rate of protein synthesis in the biosynthetic pathway, the rate of insertion into the apical membrane and the rate of endocytotic processes. Currently, we know more about down-regulation (via endocytosis) of Na^+/P_i cotransporters (in particular NaPi-lla) than about the possible regulation of the apical sorting and insertion of newly synthesized cotransporters into the brush border membrane. In the following section, we will present an overall picture of the current knowledge about the acute hormonal regulation of Na^+/P_i cotransporters, such as, e.g., by parathyroid hormone. Other important regulators of apical Na^+/Pi cotransporter abundance, such as phosphatonins, P_i diet and steroids are discussed in separate sections. Finally, a few cases in which changes of renal excretion of Pi do not match alterations of apical abundance of Na^+/Pi cotransporters will be discussed.

Changes in renal P_i excretion related to alterations of Na^+/P_i cotransporter abundance

Parathyroid hormone

The paradigm for the alteration of the apical abundance of Na^+/P_i cotransporters is represented by the regulation of the NaPi-lla protein by parathyroid hormone (PTH) (Fig. 2a). Upon stimulation of PTH receptors, a decrease of apical NaPi-lla abundance occurs within minutes, without changes in mRNA levels [4, 58]. In contrast, several hours are required for a PTH-induced redistribution of NaPi-llc [90, 103]. This indicates that (at least in rodents) acute adjustment of renal P_i reabsorption by PTH occurs mostly by a redistribution of NaPi-lla. So far, no regulation of Pit-2 by PTH in proximal tubules has been described.

Stimulation of PTH receptors leads to a rapid internalization of NaPi-lla via an endocytotic machinery located at the intermicrovillar clefts that was not associated with fluid phase uptake, but was shown to be mechanistically similar to a receptor-mediated process that involves clathrin-coated vesicles [4]. Although putative endocytic motifs such as tyrosine- or di-leucine-based motifs are contained within the amino acid sequence of NaPi-lla, mutations of these motifs did not affect the retrieval of NaPi-lla [50]. Instead, a dibasic amino acid motif (KR), most likely localized within

an intracellular loop, was found to be required for internalization and degradation of NaPi-lla [56]. The absence of this KR motif in the NaPi-llc protein could explain the different endocytic rates of the NaPi-lla and NaPi-llc cotransporters after PTH stimulation. It is of interest that the peroxisomal farnesylated protein PEX19 was reported to interact with NaPi-lla via the KR motif, yet the precise role of PEX19 in the trafficking of NaPi-lla has not been elucidated [54].

Unlike megalin [28], internalized NaPi-lla is not delivered to recycling subapical endosomes but, instead, is routed from the early endosomes to the lysosomes where it is degraded [4, 59, 89] (Fig. 2a). No lysosomal association of NaPi-llc was detected after stimulation with PTH [90, 103], which again indicates that any intracellular trafficking of NaPi-llc after hormonal stimulation differs from that of NaPi-lla.

Several observations suggest that the final reaction(s) of the PTH-induced signaling cascades leading to NaPi-IIa endocytosis may affect the affinity of the cotransporter for its interacting proteins. The signaling cascades involved in the action of PTH have been well described. Receptors for PTH are localized both at the apical and the basolateral site of proximal tubular cells and differ with respect to their activation by different PTH fragments and formation of intracellular second messengers [41]. Stimulation of basolateral PTH receptors by 1–34 PTH (via Gs) provokes an increase of the intracellular cAMP concentration and activation of protein kinase A-dependent pathways, whereas stimulation of apical PTH receptors by 1-34 PTH or 3-34 PTH (via Gq) results in the hydrolysis of PIP_2 and activation of pathways involving protein kinase C activity. Experiments performed on isolated rat proximal tubules indicated that stimulation of both, apical and basolateral PTH receptors, elicits a down-regulation of NaPi-lla [119]. Apical PTH receptors are part of a signaling complex scaffolded by NHERF1 that also includes phospholipase PLCß2 [77]. The importance of such a spatial arrangement for the hormonal regulation of the abundance of NaPi-lla was demonstrated in experiments using kidney slices derived from NHERF1 $^{-/-}$ mice. The coupling of apical PTH receptors to the down-regulation of NaPi-lla was completely abolished in the absence of NHERF1, whereas down-regulation of NaPi-lla induced by a stimulation of basolateral PTH receptors (via cAMP) remained intact [22].

In addition to PKA and PKC pathways, regulation of the amount of NaPi-lla by a pathway that involves protein kinase G should also be considered. Early studies documented that part of the renal response to the atrial natriuretic factor (ANF) includes changes of Na^+/P_i cotransport [48]. Later, it was demonstrated that increases of cGMP concentrations induced by ANP or by nitric oxide (NO) resulted in a down-regulation of NaPi-lla [3].

An involvement of MAPK (ERK1/2) kinases in the PTH action has been suggested from experiments performed with opossum kidney (OK) cells [67]. Moreover, in ex vivo studies, using mouse kidney slices, down-regulation of NaPi-lla initiated by 1-34 PTH was partially prevented by the ERK1/2 inhibitor PD098059 [5]. Similarly, down-regulation of NaPi-lla was partially inhibited by this blocker when PKC or PKG pathways were pharmacologically activated separately. In contrast, inhibition of ERK1/2 completely abolished endocytosis of NaPi-lla in response to direct activation of PKA [5]. Based on these observations, we can envisage a central role of the MAPK pathway as a point where the different pathways (PKA, PKC, and PKG) may converge (Fig. 2a). Interestingly, inhibition of renal P_i reabsorption by FGF-23 (see below) was also reported to involve activation of the MAPK kinase pathway [129].

Is MAPK always required for down-regulation of NaPi-lla? Investigations related to the phosphaturic effect of dopamine [39] demonstrated that dopamine elicits a down-regulation of NaPi-lla via stimulation of apical dopamine receptors D1 [2]. This D1-receptor-mediated effect on NaPi-lla abundance was completely prevented by a blocker of PKA (H89) but not by inhibiting ERK1/2 MAPK kinases, which indicates that cAMP dependent pathways relevant for down-regulation of NaPi-lla may differ, depending on which receptor is activated (Fig. 2a).

The subcellular location of NHERF proteins remains unaffected after PTH treatment. The implication of different kinases in the signaling cascades described above suggest that the stability of NaPi-IIa within apical heteromultimeric protein complexes, such as the one scaffolded by NHERF1, may be altered by protein phosphorylation. Studies addressing this question revealed that NaPi-lla was not phosphorylated either basally or upon stimulation by PTH [34]. However, increased phosphorylation of NHERF1 in response to PTH has been observed in murine kidney slices and suggested that phosphorylation of NHERF1 could induce a dissociation of the NaPi-lla/NHERF1 complex [34]. Recently, a more detailed study demonstrated that after activation of PKC and PKA pathways, a serine residue (Ser77) within the first PDZ domain of NHERF1 was phosphorylated [128] (Fig. 2c). Phosphorylation of Ser77 upon activation of PKC appeared to occur more directly compared to the PKA effect. Because NaPi-lla interacts with the first PDZ domain of NHERF1 [43, 44] and Ser77 is located within the predicted binding domain, phosphorylation of Ser77 is thought to induce a conformational change resulting in a lowered affinity of the NaPi-lla/NHERF1 interaction. In this respect, it is interesing to note that truncation of the PDZ binding motif of the CFTR channel increased its diffusional mobility within the membrane [47]. Similarly, by promoting phosphorylation

of NHERF1, PTH (and possibly other factors) could induce an increase of the mobility of NaPi-lla along the microvillus axis that may be required for internalization at the intermicrovillar clefts.

Phosphatonins

Tumor-induced osteomalacia (TIO), autosomal-dominant hypo-phosphatemic rickets (ADHR), recessive hyperphosphatemic rickets (ARHR) and X-linked hypophosphatemic rickets (XLH) are associated with phosphaturia and defects in bone metabolism [126]. Associated with these diseases, various new factors, termed phosphatonins, have been identified and were shown to be involved in the observed renal P_i wasting. So far, the list of phosphatonins comprises fibroblast growth factor 23 (FGF-23), secreted frizzled related protein-4 (sFRP-4), matrix extracellular phosphoglycoprotein (MEPE), and fibroblast growth factor 7 (FGF-7). The roles of phosphatonins in whole body P_i-homeostasis under pathophysiological conditions has recently been reviewed [12, 17, 55, 91, 117, 126]. However, apart from their roles in disease conditions, phosphatonins may exert regulatory functions on renal P_i reabsorption also under normal physiological conditions, whereby interference by other factors cannot be excluded [97, 106, 107, 109]. Overall, little is known about the signaling pathways of phosphatonins in proximal tubule cells that result in alterations of the abundance of Na^+/P_i cotransporters. Current knowledge about the cellular mechanisms is briefly discussed below.

Fibroblast growth factor FGF-23 FGF-23 was identified in patients with autosomal dominant hypophosphatemic rickets (ADHR) by positional cloning [30]. The phosphaturic effect observed at elevated serum levels of FGF-23 correlated with a decrease of the abundance of NaPi-lla and NaPi-llc [79, 80]. Moreover, $FGF23^{-/-}$ mice display the converse behavior with increased P_i reabsorption and hyperphosphatemia [107]. In ex vivo studies, using isolated proximal tubules, FGF-23 was reported to reduce NaPi-lla abundance [6].

FGF-23 likely activates one of the known c-splice isoforms of FGF receptors (FGFR). Some in vitro studies suggested the interaction of FGF-23 with FGFR's 1c, 3c , and 4c; however, the identity of the FGFR responsible for the action of FGF-23 in proximal tubules is currently unknown. FGF-23 receptors belong to the family of receptor tyrosine kinases, and its activation appears to be dependent on the presence of sugar chains such as those contained in heparin or klotho [65, 66, 74]. It is significant that klotho (see below) is required for the conversion of canonical FGFR's into functional FGF-23 receptors [122]. The importance of klotho for FGF-23 action was further suggested from observations made using a $klotho^{-/-}$ mouse model that develops a phenotype similar as $FGF\text{-}23^{-/-}$ mice [102]. In $klotho^{-/-}$ mice, the increase of serum P_i concentration could be correlated with increased abundances of NaPi-lla and NaPi-llc despite a massive increase of FGF-23 serum levels.

Studies in OK cells provided evidence that the signaling pathway activated by FGF-23 involves MAPK/ERK1-2 kinases [129]. Similarly as described for the PTH-induced signaling pathway (see above), the action of FGF-23 was blocked by inhibitors of ERK1/2. In addition, in isolated proximal tubules, FGF-23 was reported to provoke an increase of the production of prostaglandin PGE2 that was also suggested to occur via the MAPK/ERK pathway [111].

Secreted frizzled-related protein-4 Secreted frizzled-related protein-4 (sFRP-4) is overexpressed in TIO patients presenting renal P_i wasting [for review see 12]. Infusion of sFRP-4 into rodents was shown to provoke phosphaturia that could be correlated with a decrease of NaPi-lla [10]. sFRP-4 is an antagonist of the Wnt pathway and, indeed, after infusion of sFRP-4, a decrease of the phosphorylated form of ß-catenin was detected. However, the time course and the precise mechanism by which sFRP-4 leads to down-regulation of NaPi-IIa needs to be explored further.

Matrix extracellular phospho-glycoprotein and fibroblast growth factor 7 Overexpression of two other factors matrix extracellular phospho-glycoprotein (MEPE) and FGF-7, has been noted in tumors of TIO patients as well [for review see 12]. MEPE was also identified in tissue from a patient with oncogenic hypophosphatemic osteomalacia [95]. Conflicting results have been reported with respect to a direct action of MEPE on renal P_i handling. A recent study demonstrated that in rats, infusion of recombinant MEPE elicited an increase of P_i excretion [35]. Together with data obtained from cell culture experiments [96], it appears that MEPE indeed participates in the control of proximal P_i reabsorption. However, the precise mechanisms of the action of MEPE are not known. Also, further investigation will be required to establish if other factors regulating P_i homeostasis also interact with the renal action of MEPE.

Only minimal information is available at present about FGF-7 regarding its role in renal P_i wasting as observed in TIO patients. Based on studies performed with OK-cells, FGF-7 inhibits Na^+/P_i cotransport [25].

Klotho

In addition to interaction with FGF receptors (see above), klotho may exert direct effects on the abundance of Na^+/P_i cotransporters in proximal tubules. The extracellular domain of klotho, which is anchored by a single transmem-

brane segment, shows ~40% homology to ß-glycosidase family-1 enzymes and thus may enyzmatically alter sugar moieties of membrane proteins [118]. In kidneys, klotho is expressed in distal convoluted tubules [65]. Interestingly, the extracellular domain of klotho is shed from the membrane and is detected in the blood, which indicates that this domain may act as a humoral factor [53, 65, 85]. Evidence for a direct action of klotho on NaPi-lla abundance was reported from in vitro experiments using microperfused proximal tubules and isolated BBMV's [52]. Most interestingly, incubation of BBMV's with klotho reduced Na^+/P_i cotransport activity, an effect most likely was due to a change in the glycosylation of NaPi-lla itself. These observations suggest that modification of the glycosylation moieties of NaPi-lla may affect its apical stability, similar to that described for the calcium channel TRPV5 [27].

Dietary P_i intake

It has long been known that changes in the dietary intake of P_i affect renal excretion of P_i. Interestingly, the capacity of renal P_i reabsorption adapts to altered intake of P_i within less than an hour (acute adaptation) and remains adjusted during prolonged intake of dietary P_i (chronic adaptation) [71, 73].

Adaptation to altered P_i diet is independent of PTH, vitamin D3, and growth hormones [19, 24, 71, 120]. However, analysis of the mechanisms involved in such dietary adaptation can be complicated by the involvement of other factors. For example, serum levels of FGF-23 were reported to be dependent on the state of phosphatemia [127]. Furthermore, studies performed with diabetic rats indicated that insulin may have a permissive effect on the adaptive response to low P_i diet as the increase of apical Na^+/P_i cotransport was abolished in these rats [1]. Also, lowered plasma levels of P_i may affect the rates of metabolic pathways of proximal tubular cells, such as for example of gluconeogenesis [64].

Within the first four hours after ingestion of a low P_i diet, brush border Na^+/P_i cotransport increases due to an increase of the amount of NaPi-lla [26, 71, 73]. Rapid up-regulation induced by low P_i diet was also observed for Pit-2 but not for NaPi-llc [79 and Ravera S et al., unpublished observations]. Whereas acute changes of NaPi-IIa abundance are independent of transcriptional regulation, controversial findings regarding the chronic regulation have been reported. In weaning mice, a chronic low P_i diet elevates the amount of NaPi-lla mRNA in addition to protein abundance. This effect was ascribed to a P_i responsive element located within the promoter region of the Npt2 gene [60]. However, in adult mice fed chronically with a low P_i diet an increase of NaPi-llc mRNA but not of NaPi-lla mRNA was observed [76].

The signaling mechanisms involved in dietary regulation are not fully understood. The rapid increase of brush border Na^+/P_i cotransport was not blocked by cycloheximide, which suggests that de novo synthesis of Na^+/P_i cotransporters might not be necessary [73]. Yet, so far, there is no clear evidence for the existence of an intracellular, subapical pool of Na^+/P_i cotransporters that could explain the rapid adaptive phenomenon. Based on the observation that the fall in the serum level of P_i after ingestion of a low P_i diet precedes up-regulation of Na/Pi cotransport [73], one might hypothesize the existence of one or more sensing mechanism(s) for P_i that eventually may be located in the proximal tubular cells.

Renal excretion of P_i decreases after ingestion of a diet rich in P_i due to a reduction of apical Na^+/P_i cotransporter abundance. High P_i diet induces internalization of both NaPi-lla and NaPi-llc, yet by distinct mechanisms. Whereas the amount of NaPi-lla in BBMV's was decreased after 2 h, reduction of NaPi-llc was detectable only after 4 h. As found for the PTH action, internalized NaPi-lla (by high P_i diet) is routed to the lysosomes, whereas NaPi-llc is redistributed from the BBMV to a subapical compartment and does not undergo lysosomal degradation [59, 71, 101].

Acute regulation of renal P_i excretion has recently been investigated after application of a P_i bolus directly into the duodenum of rats [13]. This maneuver elicited an increase of renal excretion of P_i already within 15 min. It is noteworthy that this response preceded changes in the plasma P_i, was independent of PTH and the phosphatonins FGF-23 and sFRP-4 and was not observed in animals with denervated kidneys. These data suggest that renal P_i excretion may be part of an intestinal–renal axis that may include a novel (humoral) factor originating from the duodenal mucosa.

Steroid hormones

The influence of steroid hormones on renal Na^+/P_i cotransporters has been studied under chronic conditions. Although chronically administered, steroids could act via genomic mechanisms, nongenomic effects cannot be excluded. Furthermore, it has to be considered that other factors may be involved in the action of a particular steroid.

Glucocorticoids In addition to other effects in proximal tubules, such as stimulation of the rate of gluconeogenesis, glucocorticoids are also involved in the regulation of the renal handling of P_i [36, 39, 108]. In kidneys from rats that were treated chronically with dexamethasone, the amount of NaPi-lla protein and mRNA was decreased, in agreement with increased excretion of P_i [72]. Furthermore, an inverse correlation between Na^+/P_i-cotransport in BBMV's and glucosylceramide and sphingolipid content was reported in

these studies. It remains to be determined if glucocorticoids may affect, besides NaPi-lla, other apical Na^+/P_i cotransporters as well.

Estrogen Chronic treatment of ovarectomized rats with estrogen results in hypophosphatemia and hyperphosphaturia. Renal wasting of P_i observed under these experimental conditions has been explained by reduced Na^+/P_i cotransport in BBMV's due to a decrease of NaPi-lla abundance [8, 37]. Moreover, estrogen treatment was shown to decrease the abundance of NaPi-lla mRNA, whereas NaPi-llc mRNA was unaffected. It remains to be determined if the reported effect of estrogen on NaPi-lla is a direct or indirect one. Although the effect on NaPi-lla was reported to be independent of PTH [37], the reduction in NaPi-lla abundance could result from an indirect action of estrogen on dopamine receptors and/or the metabolism of dopamine in proximal tubules [68].

1,25(OH)2-Vitamin D3 (VitD3) Although the profound effects of VitD3 on the metabolisms and homeostasis of calcium and phosphate are well described [20, 108], conflicting results about direct effects of VitD3 on P_i reabsorption in proximal tubules have been reported.

In thyroparathyroidectomized (TPTX) rats, chronic administration of VitD3 was reported to inhibit P_i reabsorption in proximal tubules [82]. Although interference by PTH was excluded in this study, more recent data suggest that VitD3 action on renal P_i handling could be indirectly, involving altered serum levels of phosphatonins [109]. For example, in intact rats, repeated injections of VitD3 resulted in an increase of serum levels of FGF-23 [106]. Furthermore, FGF-23 impairs the renal metabolism of VitD3 by suppressing the expression of 25-hydroxyvitamin D 1α-hydroxylase mRNA and by stimulation of the expression of 25-hydroxyvitamin D-24 hydroxylase mRNA [88, 107].

A VitD3 responsive element has been identified in the promoter region of the human NaPi-lla gene, suggesting that regulation of renal P_i reabsorption by VitD3 may occur at the transcriptional level [114]. However, in VitD3 receptor and 1,α-OHase$^{-/-}$ mice, the abundance of the NaPi-lla protein in BBMV was reported to be similar as the one of wild-type mice [24, 100], which indicates that at least in mice, the NaPi-lla abundance may not be controlled by VitD3 dependent transcriptional mechanisms.

Changes in renal P_i excretion not related to alterations of Na^+/P_i cotransporter abundance.

Apart from the hormonal and metabolic settings that result in a change of the abundance of Na^+/P_i cotransporters as discussed above, a few observations indicate that altered renal Pi excretion can occur also without parallel changes of the amount of apical Na/Pi cotransporters.

Potassium depletion

In rats depleted of potassium, P_i excretion was increased and was in agreement with a decreased Na^+/P_i cotransport in isolated BBMV's. However, this change of Na^+/P_i cotransport was not correlated with a reduced abundance of NaPi-lla, but instead by increased amounts of NaPi-lla. [131]. These contradictory findings were explained by an alteration of the lipid composition, (e.g., glucosylceramide), and altered fluidity of the brush border membrane. Similarly, altered rates of Na^+/P_i cotransport occurring during aging and chronic adaptation to a dietary restriction of P_i may also be explained partially by a change of membrane fluidity due to an altered ratio of lipids to cholesterol in the brush border membrane [69, 81].

Metabolic acidosis

Metabolic acidosis provokes an increase of P_i excretion [11, 108]. Transcriptome analysis revealed that NaPi-llc mRNA is markedly down-regulated in mice after 2 days of metabolic acidosis, less pronounced down-regulation was observed for NaPi-lla mRNA [86]. In contrast, the abundance of both proteins was increased, consistent with higher Na^+/P_i cotransport in BBMV isolated from acidotic mice compared to control animals [87]. Increased urinary P_i excretion was explained by a lower tubular pH-value that decreases the transport rates of NaPi-lla/c.

Recovery after PTH treatment

In rats injected with a single bolus of PTH, fractional excretion of P_i was maximally increased after 40 min and recovered after 120 minutes. Whereas maximal excretion of P_i correlated with a decrease of the abundance of NaPi-lla, the levels of NaPi-lla did not recover parallel to the recovery of P_i excretion [40]. These data indicate that the rate of recovery of the amounts of Na^+/P_i cotransporters after interventions that provoke their internalization is an important regulatory variable.

Circadian rhythm

Both serum concentration and renal excretion of P_i undergo circadian variations [57]. In rats, fractional excretion of P_i increased substantially between morning and late afternoon, yet, a change in the abundance of NaPi-lla was not detected [16]. Although part of this observation can be explained by an increase of serum P_i during daytime, it remains to be

shown if fluctuations of the amount of NaPi-llc and/or Pit-2 may contribute to the diurnal variations of P_i excretion.

Future directions—open questions

Other (Na)/Pi (co)transporters? Although several Na^+/P_i cotransporters have been localized at the brush border membrane of proximal tubule cells, it is still possible that additional cotransporters may be involved in P_i reabsorption along PT's. If so, it will be of interest to elucidate the relative contribution of each transporter to renal P_i reabsorption in different species. Moreover, the often postulated handling of tubular P_i in distal nephron segments remains to be clarified. Another long-standing open question is the molecular identity of the basolateral exit step of P_i.

Other phosphaturic factors? Recent investigations have added new members to the list of the factors that regulate P_i reabsorption. In the light of a systemic control of renal P_i handling by different axis (bone-kidney, intestine-kidney and eventually others) it is likely that more factors are about to come under the spotlight. Furthermore, the possibility of (a) P_i sensing mechanism(s) (P_i sensor?) remains unanswered.

Control of apical abundance? Although current data suggest that phosphorylation reactions are involved in the control of the apical abundance of Na^+/P_i cotransporters, e.g. by PTH, the signaling cascades of the diverse hormones (including the phosphatonins) that control their apical abundance remain to be elucidated. Moreover, the cellular mechanisms involved in the rapid adaptive responses to altered P_i diets need further investigation.

Trafficking of Na^+/P_i cotransporters Related to the intracellular trafficking of Na/Pi cotransporters the following questions remain unanswered:

-How are Na^+/P_i cotransporters sorted to the apical membrane?
-Which mechanisms are involved in the routing of Na^+/P_i cotransporters (specifically NaPi-lla) from the subapical compartment to the lysosomes?

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